Construction of Molecular Database for Management of Fungal Diseases Associated with *Trigonella foenumgraecum* L. (Fenugreek)

A

SYNPOSIS

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INTRODUCTION

Fenugreek *(Trigonella foenum-graecum* L.) is an annual forage legume crop. The species name *"foenum-graecum "means* "Greek hay" indicating its use as a forage crop in the past. Fenugreek is believed to be native to the Mediterranean region (Petropoulos 2002), but now is grown as a spice in most parts of the world. It is reported as a cultivated crop in parts of Europe, northern Africa, west and south Asia, Argentina, Canada, United States of America (USA) and Australia (Fazli and Hardman 1968; Edison 1995; AAFRD 1998; Petropoulos 2002). India is the leading fenugreek producing country in the world (Edison 1995).

Fenugreek (*Trigonella foenum-graecum*) is a plant belongs from family Fabaceae. Fenugreek is used both as a herb (the leaves) and as a spice (the seed, often called Methi in Urdu/Hindi/Nepali). Fenugreek is regarded as the oldest known medicinal plant in recorded history (Lust 1986).Fenugreek has been referred to as a medicinal herb both in Indian Ayurvedic and traditional Chinese medicines (Tiran 2003). Ancient literature, religious scripture, travel records and anecdotes from different continents and from different periods of human history, record a wide variety of medicinal properties associated with fenugreek (Lust1986). Medicinal uses vary from wound-healing to bust enhancement and, from promotion of lactation in weaning mothers, to its use as a sex stimulant or aphrodisiac (Petropoulos 2002; Tiran 2003). The leaves and sprouts are also eaten as vegetables. The plant is cultivated worldwide as a semi-arid crop and is a common ingredient in many curries.

Plant Description:

Kingdom	*Plantae*
Subkingdom	*Tracheobionta*

Superdivision	*Spermatophyta*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Subclass	*Rosidae*
Order	*Fabales*
Family	*Fabaceae*
Genus	*Trigonella* L.
Species	*Trigonella foenum-graecum* L.

Major fenugreek producing countries are Nepal, India, Pakistan, Bangladesh, Argentina, Egypt, France, Spain, Turkey, Morocco and China. India leads the world in fenugreek production, producing70-80% of the global export (Edison 1995). India is the largest producer of fenugreek in the worldwide where Rajasthan, Gujarat, Uttaranchal, Uttar Pradesh, Madhya Pradesh, Maharashtra, Haryana and Punjab are the major fenugreek producing states. Rajasthan produces the lion's share of India's production, accounting for over 80% of the nation's total fenugreek yield. Qasuri Methi, more popular for its appetizing fragrance, comes from Qasur, Pakistan, and regions irrigated by the Sutlej River, in the Indian and Pakistani states of Punjab. A major part of fenugreek production in Rajasthan is carried out in Sikar which accounts to 80% of the total output, followed by areas of Nagor, Chittor, Bhilwara and Jhunjhunu. Ideal sowing time for fenugreek crop in Northern India is last week of October to first week of November. In southern parts of the country, fenugreek is grown both as a Kharif crop and as a Rabi crop. Ideal sowing time for kharif crop is second fortnight of June to July-end and ideal sowing time for rabi crop is

first fortnight of October. The average yield of *Trigonella foenumgraecum* crop is however low due to lack of superior varieties, scientific crop production technology and vulnerability to diseases like **Wilt, Blight and Powdery mildew incited by *Fusarium oxysporum* f.sp.*cumini*, *Alternaria burnsii* and *Erysiphe polygoni*,** respectively. While efforts are being made to evolve better varieties and agronomical practices through AICRPs and NRCs but efforts to combat these diseases are meager. All of these three diseases, cause serious losses in seed yield particularly, if the weather conditions are congenial for disease development. Though the disease has been investigated at field level and laboratory level but not much could be achieved as far as resistance is concerned. Thus with non-availability of resistance source in the germplasm, it is important to undertake studies to develop suitable genotype using biotechnological tools.

Certain difficulties have been encountered for the management of bacterial/fungal diseases in plants because of scanty availability of known and recommended bactericides in the market. In addition, these antibiotics are costly and not fully efficacious in some cases. In fact, during last two decades, problems of resistance against streptomycin have increased as well as some new important bacterial pathogenic strain have been identified and documented. Consequently, there is an urgent need to search for economic and eco-friendly alternative strategies for the management of fungal diseases. Recently greater emphasis on stable source of resistance and other useful control measures viz., biological, cultural and botanicals have been laid. Besides, various biotechnological approaches have also opened new vistas in the management of fungal diseases.

Until serological techniques were developed, the only reliable methods available for identification of fungi and bacteria were isolation in culture and performing morphological and biochemical tests. Serological techniques allowed rapid presumptive diagnosis of bacterial

diseases (Hampton *et al*., 1990). More recently, a number of approaches have been developed to study molecular microbial diversity. These include DNA re-association, DNA–DNA and mRNA: DNA hybridization, DNA cloning and sequencing, and other PCR-based methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA).

PCR targeting the 16S rDNA has been used extensively to study prokaryote diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationships (Pace, 1996, 1997, 1999). 18S rDNA and internal transcribed spacer (ITS) regions have extensively been used for prokaryotes. In these methods, DNA is extracted from the environmental sample and purified, target DNA (16S, 18S or ITS) is amplified using universal or specific primers and the resulting products are separated in different ways.

IMPORTANCE OF STUDY

- The importance of present investigation is to find improved strategies for characterization, conservation and to increase the utilization of plant & microbial genetic resources.
- As agricultural practices become more sustainable, there is an increasing need for ecologically sound methods of disease control.
- Biological control, which exploits the natural antagonistic activity of certain root-colonizing bacteria against fungal pathogens, is one such approach.
- Biological control agents often perform inadequately under field conditions, however, this has impeded acceptance of the technology as an alternative to chemical pesticides.

Due to the agro climatic conditions of different regions, the genotype of pathogen may vary.

- The effective database of pathogen may help in selecting best suitable agrochemical and biocontrol agent to prevent crop losses.

OBJECTIVES

The present study has been planned keeping in mind the following objectives:-

1. Collection of *Trigonella foenum-graecum* samples from growing areas of Western Rajasthan.
2. Isolation, Purification, Identification & Pathogenicity test of fungal isolates of above *Trigonella foenum-graecum* samples.
3. Efficacy of various antifungal agents against fungal diseases of *Trigonella foenum-graecum* under in vitro control conditions.
4. Identification of genetic diversity among various fungal isolates using molecular markers.
5. Construction of database based on genetic variants of fungal isolates.

REVIEW OF LITERATURE

National Status

Plant references about disease

Use of organic and inorganic fertilizers, farmyard manure, nitrogen and phosphorus have been found to be effective in increasing fenugreek yield (Detoroja *et al.*1995; Khiriya and Singh 2003; Yadav and Kumawat 2003). Abd-Ala and Omar (1998) reported that application of wheat straw and fungi *(Sinorhizobium meliloti, Trichoderma harzianum, Aspergillus niger* and *Chaetonium globosum)* promote nodulation, nodule efficiency and fenugreek growth under saline soil conditions. Fenugreek is a nitrogen fixing legume. Hence the seed must be inoculated with an appropriate *Rhizobium* inoculum to optimize its growth potential. The most common nodule-forming bacteria associated with *Trigonella foenum-graecum* L. is the Gram negative, aerobic, non-sporulating, rod shaped bacterium, *Rhizobium meliloti* (Subba Rao and Sharma 1968). Abdelgani *et al.* (1999) has suggested that inoculation of fenugreek with a suitable strain of *Rhizobium* can improve quality and amount of seed generated.

Fungal, bacterial, viral and insect mediated diseases are reported to be associated with considerable lowering of forage and seed yield in fenugreek and hence is a serious agronomic concern (AAFRD 1998; Fogg *et al.* 2000; Jongebloed 2004; Petropoulos 1973, 2002; Prakash and Sharma 2000).The two most common fungal diseases infecting fenugreek are ercospora leaf spot and powdery mildew (AAFRD 1998). Prakash and Sharma (2000), Petropoulos (2002) and Jongebloed (2004) have reported powdery mildew on fenugreek, caused by *Erysiphe polygoni.* Additional studies from Australia (Jongebloed 2004) have shown that yield of fenugreek can be seriously affected by blight disease caused by *Cercospora traversiana* and wilt caused by *Fusarium oxsysporum* and *Rhizoctonia solani.* In addition, other

well known fungal diseases associated with fenugreek are collar rot, leaf spot and pod spot diseases (Petropoulos 2002). Fogg *et al.* (2000) reported a bacterial leaf spot in fenugreek which was caused by *Pseudomonas syringae* pv. *syringae* in New Jersey, USA. It also has been suggested that the bacterium *Xanthomonas alfalfa* can infect fenugreek (Petropoulos 2002).Petropoulos (2002) reported that Bean Yellow Mosaic Virus, Alfalfa Mosaic Virus, Cow Pea Mosaic Virus, Soybean Mosaic Virus, Pea Mosaic Virus, Potato Virus A and Y, and Clover Vein Mosaic Virus are common viral infections of fenugreek. These viral diseases have been associated with moderate loss of seed and forage yield. Lucy (2004) reported that in Australia insects such as thrips, pod-borers and heliothis can cause serious damage to forage yield in fenugreek. Root rot by the soil borne nematode *Meloidogyne incognita,* which causes the death of immature plants has also been reported from Australia (Jongebloed 2004). Fenugreek is reported to be sensitive to mineral deficiencies (Petropoulos 1973). It has been suggested that yellowing of some fenugreek plants under field conditions is connected to probable mineral deficiencies, in particular to elements like boron, magnesium, manganese or potassium deficiency (Sinskaya 1961). Physiological diseases have been reported to be associated with early death and loss of forage and seed yield in fenugreek (Petropoulos 2002). The crop prefers faintly alkaline soil with a pH range of 8-8.5. Potash has been used to adjust soil pH to increase nutrient uptake of fenugreek (Yadav and Kumawat 2003). The application of organic and inorganic fertilizers, farmyard manures, nitrogen and phosphorus has been found to be effective in increasing fenugreek yield (Detoroja *et al.* 1995; Khiriya and Singh 2003; Yadav and Kumawat 2003). Lai *et al.* (2003) reported that optimization of sowing dates and forage cutting maximize fenugreek yield.Fenugreek is a nitrogen fixing legume. Hence the seed needs to be inoculated with an appropriate *Rhizobium* inoculum to optimize this potential (Abdelgani *et al.*

1999).The most common nodule-forming bacteria associated with fenugreek are the Gram negative, aerobic, non-sporulating, rod shaped bacterium, *Rhizobium meliloti* (Subba Rao and Sharma 1968). Abd-Ala and Omar (1998) reported that application of wheat straw and certain fungi promotes nodulation, nodule efficiency and growth of the crop under saline soil conditions. Balajee *et al.* 2005a, 2007) Clinically, identification of unknown *Aspergillus* clinical isolates to species may be important given that different species have variable susceptibilities to multiple antifungal drugs. Thus,knowledge of the species identity may influence the choice of appropriate antifungal therapy. For example, *in vitro* and *in vivo* studies have demonstrated that *A. terreus* isolates are largely resistant to the antifungal drug amphotericin B, *A. ustus* isolates appear to be refractory to azoles, and *A. lentulus* and *Petromyces alliaceus* have low *in vitro* susceptibilities to a wide range of antifungals including amphotericin B, azoles, and echinocandins . Foot rot disease caused by Rhizoctonia solani in fenugreek is one of the important diseases resulting in heavy losses in Rohilkhand. Bareilly is one of the chief production centres of crop, facilitating the bulk supply to Uttranchal and nearby states. The production of fenugreek is hampered by foot-rot disease. The fungus is soil borne and all parts of fenugreek are prone to infection (Singh et al., 2010). Application of fungicides is the only strategy for the management of fungal diseases since many years. Conventional breeding for fungal disease tolerance has not proven to be very effective in identifying a resistance source. Not only the availability of such a simple, safe and fast technique has been of great importance for diversification but also in various field of DNA analysis as evidenced by the review of literature Kumar et al. (2002) reported that plant growth promoting rhizobacterial strains belonging to fluorescent Pseudomonas were isolated from the rhizosphere of rice and sugarcane among 40 strains that were confirmed as Pseudomonas fluorescence, 18 exhibited strong antifungal activities against Rhizoctonia

bataticola and Fusarium oxysporum, mainly through the production of antifungal metabolites. Genotyping of these Pseudomonas fluorescence's strains was made by PCR- RAPD analysis, since differentiation by biochemical methods was limited. Thakuria et al. (2004) studied three groups of rhizobacteria, isolated from the rhizosphere of rice grown in acidic soils of Assam. Isolated bacteria were selected for taxonomic identification, characterization and also for screening the superior isolates to promote rice growth. RAPD analysis of the isolates indicates distinct genotypes. These bacterial strains showed differences in growth pattern, IAA production level and antibiotic resistance profile and nitrogenase activity in the inoculated roots. Satyanaryana et al. (2005) reported the diversity of microorganisms that occur in extreme environments, their adaptations and potential biotechnological applications. Both culture dependent and culture independent (molecular) methods have been employed for understanding the diversity of microbes in these environments. They observed that the extremophiles have evolved several structural and chemical adaptations, which allow them to survive and grow in extreme environments. The enzymes of these microbes, which function in extreme environments (extremozymes), have several biotechnological applications. Sharma et al. (2005) identified, isolated and characterized an entophytic bacterium related to Rhizobium/ Agrobacterium from wheat (Triticum aestivum L) roots. For genotypic characterization, isolate 24 was grown in the medium for 48 hours and total DNA was isolated using the CTAB method. The 16S rDNA was amplified using the primers 41F and 1488R. The amplified DNA was partially sequenced using forward primer 41F on an ABI prism 3700 (Applied Biosystem, USA) DNA sequencer. Mathur et al. (2008) registered more than 95% establishment in soil following treatment with various bio-inoculants namely; Glomus aggregatum, Trichoderma harazianum and Piriformospora indica whereas Azospirullum sp. (CIM-azo) and Actinomycetes sp. (CIM-actin) showed only

up to 85% plantlet establishment of micro-cloned plantlets of Chlorophytum borivilianum. The un-rooted shoots were also treated with these bio-inoculants, for in vivo root induction and increased survival rate/establishment frequency when transferred to soil. The un-rooted shoots also showed in vivo rooting (50%) when treated with mycorrhiza Glomus aggregatum (VAM) and Trichoderma harazianum. The genetic fidelity testing of micro-cloned, bio-hardened progeny based on a RAPD analysis using 40 random decamer DNA primers indicated a strong uniformity in relation to the parent genotype.

International Status

Kuske et al. (1998) observed efficient nonselective methods to obtain DNA from the environment, which are needed for rapid and thorough analysis of introduced microorganisms in environmental samples and for analysis of microbial community diversity in soil. A small-scale procedure to rapidly extract and purify DNA from soils was developed for field use. They found that bead mill homogenization step was effective for DNA extraction and the hot-detergent bead mill procedure was simplified and miniaturized. Dalmastri et al. (1999) studied Burkholderia cepacia populations associated with Zea mays. The genetic diversity among B. cepacia isolates were analysed by the random amplified polymorphic DNA (RAPD) technique, using the 10-mer primer AP5. The analysis of molecular variance (ANOVA) method was applied to estimate the variance components for the RAPD patterns. Dendrogram showed bacterial population with frequent recombinations and a non-clonal genetic structure. The dendrograms were also in agreement with the ANOVA results. Dunbar et al. (1999) described the levels of bacterial community diversity in four arid soils and compared them by cultivation and 16S rRNA gene cloning. They found the total of 498 phylotypes among the 16S rRNA clones. While, 34 phylotypes occurred among the cultivated isolates. They investigated the phylotype richness,

frequency distribution and composition of the 4-culture collection by using the variety of diversity indices. Gelsomino et al. (1999) studied bacterial community structure by using DNA extraction followed by molecular fingerprinting. Total community DNA was extracted and purified by a direct method, which yielded amplifiable DNA of high molecular weight for all soils. A variable region of the 16S rRNA genes was then amplified by PCR with bacterial primers resulting in a mixture of amplicons separable via denaturing gradient gel electrophoresis (DGGE). The DGGE profiles of soil were indicative of dominant of soil bacterial types.

Theron and Cloete (2000) performed molecular techniques for determining structure and function of microbial diversity in natural ecosystem. They observed that molecular approaches based on 16S rRNA sequence analysis allow direct investigation of the community structure, diversity and phylogeny of microorganisms in almost any environment. While quantification of the individual types of microorganisms or entire microbial communities may be addressed by nucleic acid hybridization techniques.

Dunbar et al. (2000) proved the ability of terminal restriction fragment profiles of 16S rRNA genes to provide useful information about the relative diversity of complex microbial communities and TRF analysis is an excellent method for rapidly comparing the relationships between bacterial communities in environmental samples. This method is interchangeable with other molecular techniques and useful for rapid analysis of replicate samples in field- scale studies. Flore et al. (2001) used a combination of multiple techniques to the identification of bacterial strains isolated from clinical and natural environment. All isolates were examined by means of rec. A based PCR assays and RFLP specific for these genomovars and species. A combination of different molecular techniques including SDS- PAGE of whole cell protein,

RFLP of 16S r DNA and rec A genes, and rec. A – Based PCR assay can be very helpful to assess the species and genomvar composition of a natural population of *B. cepacia* complex.

Bridget et al. (2004) suggested that the study of most dwarf soil microbes requires culture-independent techniques. They used sodium pyrophosphate for eluting the DNA and filtered with a 0.45µm pore size filter. Filtrate DNA was extracted and PCR amplified by using universal bacterial and archael 16S rDNA primers, cloned, RFLP–screened and sequenced. Dwarf archaea and bacteria were present in the initial filtrate and in the cultures. Grouped dwarf bacteria into four bacterial phyla; Proteobacteria, Firmicutes, Actinobacteria and the TM-7 group. They observed that several sequences showed no close relationship to any microorganisms that have been grown in culture.

Beeja et al. (2004) emphasized on sampling of wild microorganisms leading to the discovery of new species and novel metabolites. Exploring bacterial diversity is typically done amplifying rRNA genes, in particular 16S rRNA genes from DNA samples isolated from a habitat. 16S rRNA genes are considered standard because they are thought to be conserved across vast taxonomic distance.

Yeates et al. (2006) studied various methods for microbial DNA extraction from soil for PCR amplification and observed that amplification of DNA from soil is often inhibited by co-purified contaminations like organic matter especially of humic acids. DNA extracted using sonication was more degraded than for the other methods and the bead beating method performed better in comparison to other methods. This method is more likely to result in effective lyses of all soil organic matter, reduced co-extraction of inhibitors and provides sufficient amount of DNA for PCR amplification.

METHODOLOGY

- Collection of materials: Soil samples with microflora & plant material from different regions.
- Isolation of fungal pathogens from rhizosphere of fenugreek on the basis of primary morphological characteristics & biochemical tests for their pathogenic activity.
- Study of pathogenic properties of isolated fungi in pot level for different parameters like germination, root length, their fresh and dry weights, percent root infection.
- Isolation of DNA from fungal pathogens.
- ITS RNA sequence based diversity estimation of isolated fungus.
- Sequence alignment and phylogenetic analysis using Ntysis Pc 2.02e.
- Construction of database based on genetic variants of fungal isolates.

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